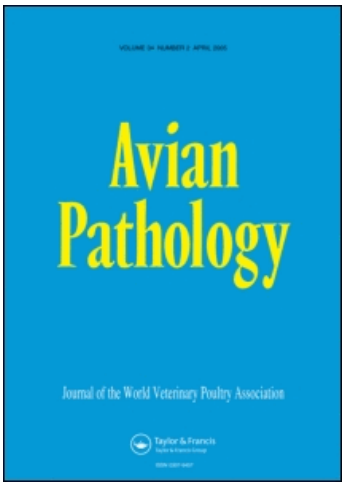

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Functions exerted by the virulence-associated type-three secretion systems during *Salmonella enterica* serovar Enteritidis invasion into and survival within chicken oviduct epithelial cells and macrophages

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Salmonella enterica serovar Enteritidis (SE) infection of chickens is a major contributing factor to non-typhoidal salmonellosis. The roles of the type-three secretion systems (T3SS-1 and T3SS-2) in the pathogenesis of SE infection of chickens are poorly understood. In this study, the functions of T3SS-1 and T3SS-2 during SE infection of primary chicken oviduct epithelial cells (COEC) and macrophages were characterized. The T3SS-1 and T3SS-2 mutants (*sipB* and *ssaV*), impaired in translocation and secretion, respectively, were significantly less invasive than their wild-type parent strain. The genes encoding effector proteins of T3SS-1 (SipA, SopB, and SopE2) and T3SS-2 (PipB) contributed equally to the entry of SE into COEC. The *sipA* mutant had reduced survival and the *pipB* mutant had enhanced replication in COEC. Mutations in the T3SS-2 genes *ssaV* and *pipB* reduced the survival of SE in chicken peripheral blood leukocyte-derived macrophages (PBLM), but not in the established chicken macrophage cell line HD11. A mutation in the *ssaV* gene also abolished SE-induced PBLM death between 1 h post-inoculation and 4 h post-inoculation. This study has shown that both T3SS-1 and T3SS-2 are required by SE to invade COEC; that SipA and PipB are necessary for the survival of SE in COEC and chicken PBLM, respectively; and that T3SS-2 triggers PBLM death during the early stages of SE infection, and this process does not depend on PipB.

Introduction

Salmonella enterica serovar Enteritidis (SE) is a major aetiological agent of non-typhoidal salmonellosis, accounting for 18.6% of all cases in the US (CDC, 2007). Consumption of SE-contaminated poultry products remains a risk factor for human SE outbreaks (CDC, 1996; Guard-Petter, 2001; Humphrey, 2006). Epidemiological studies demonstrate that contamination of raw poultry products with *Salmonella* is attributable to cross-contamination in the hatchery from infected or contaminated fertilized eggs to uninfected chicks (Cox *et al.*, 2000; Marcus *et al.*, 2006). Chickens older than 1 week of age are generally resistant to *Salmonella* infection, except with avian-adapted serovars (Vugia *et al.*, 2004; Humphrey, 2006). SE colonizes the intestinal tract and the reproductive tract of laying hens without causing overt clinical signs. The organisms have been isolated from preovulary follicles, oviduct secretions and oviduct epithelium, with the isthmus of the oviduct being the predominant colonization site of SE (Hoop & Pospischil, 1993; Okamura *et al.*, 2001; De Buck *et al.*, 2004). Although ovarian/oviduct infection is known to be the major route by which egg contents become infected, the molecular mechanisms underlying SE persistence in the chicken reproductive tract is largely unknown.

S. enterica pathogenicity island-1 (SPI-1) and SPI-2 encode two virulence-associated type-three secretion systems: T3SS-1 and T3SS-2, respectively (Hensel *et al.*, 1998; Hueck *et al.*, 1995). A functional T3SS comprises regulatory proteins, structural proteins forming a needle-like apparatus, translocases necessary for the delivery of effectors, and secreted effectors that modulate host cellular events (Hensel *et al.*, 1997; Pfeifer, *et al.*, 1999; Ruiz-Albert, *et al.*, 2003).

T3SS-1 is mainly associated with bacterial invasion of the intestinal epithelium and enterocolitis via the concerted action of effector proteins (Zhou *et al.*, 1999; Zhou & Galán, 2001; Zhang *et al.*, 2002, 2003). SopB and SopE1/SopE2 activate the Rho family GTPases, CDC42 and Rac1, leading to actin cytoskeleton rearrangement, macropinocytosis and bacterial entry (Norris *et al.*, 1998; Bakshi *et al.*, 2000; Mirold *et al.*, 2001). SipA lowers the critical concentration of actin monomers required for polymerization, which facilitates host

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cell invasion (Zhou *et al.*, 2001). Two other effector proteins, SopA and SopD, contribute to invasion of polarized epithelial cells by unknown mechanisms (Raffatellu *et al.*, 2005). Another important T3SS-1 protein, SipB, functions as a translocase for these effector proteins, and as an effector causing macrophage cell death (Collazo & Galán, 1997; Watson *et al.*, 2000).

T3SS-2 is responsible for the establishment of systemic infection by promoting the intracellular survival of salmonellae in macrophages. The effector proteins translocated by T3SS-2 inhibit the fusion of salmonella-containing vacuoles (SCVs) with vesicles of the endocytic pathway, and prevent NADPH oxidase localization and oxyradical formation at the phagosomal membrane (Gallois *et al.*, 2001; Chakravortty *et al.*, 2002). The SsaJ, SsaM, SsaV and SsaU proteins are the structural components of the T3SS-2 system, whereas the SseB, SseC and SseD function as a translocon for effector proteins, such as PipB and SifA (Hensel *et al.*, 1997; Ruiz-Albert *et al.*, 2003). Mutations in the genes encoding the secretion apparatus, translocases or certain effectors result in attenuated phenotypes in terms of either invasion or intracellular replication (Wood *et al.*, 1998; Shea *et al.*, 1999; Zhang *et al.*, 2003).

Much of our understanding of the functions of T3SSs of *S. enterica* is based on experimental systems involving serovar Typhimurium and mammalian hosts or cell cultures. The exact contributions of T3SS-1 and T3SS-2 to SE colonization of chickens are largely unknown and are assumed to resemble those of the related serovars in mammalian models. Several studies suggest that T3SS-1 is required by Typhimurium and SE to colonize the chicken intestine (Morgan *et al.*, 2004; Bohez *et al.*, 2006; Jones *et al.*, 2007). Suppression of *hilA*, a transcriptional regulatory protein of T3SS-1, by addition of medium-chain fatty acids (such as caproic acid) to the feed of chicks led to reduced colonization of the caeca and internal organs by SE (Van Immerseel *et al.*, 2004). A recent study suggested that inactivation of *ssrA*, a regulator of T3SS-2, rendered SE unable to successfully colonize the chicken oviduct (Bohez *et al.*, 2008). In this study, we have determined the roles of individual effectors of T3SS-1 and T3SS-2 in SE invasion and intracellular survival in chicken oviduct epithelial cells and macrophages.

Material and Methods

Bacterial strains and growth conditions. The SE strains and plasmids used in this study are presented in Table 1. SE338 was originally isolated from egg-associated field outbreaks (Sheela *et al.*, 2003). A spontaneous nalidixic acid-resistant strain of SE 338, designated ZM100, was generated by serial passages in trypticase soy broth (TSB) containing increasing concentrations of nalidixic acid. Bacteria were grown aerobically in TSB or on Luria–Bertani (LB) agar plates at 37°C. If appropriate, antibiotics were added at the following concentrations: chloramphenicol, 30 μg/ml; ampicillin, 100 μg/ml; nalidixic acid, 50 μg/ml.

Construction of mutants. Mutant SE strains—including ZM101 (*sipB*), ZM102 (*ssaV*), ZM103 (*sipA*), ZM104 (*sopB*), ZM105 (*sopE*2), ZM106 (*pipB*), ZM108 (*sseB*) and ZM109 (*sseC*)—were constructed by inactivating target genes as described previously (Zhang *et al.*, 2002). In brief, an internal fragment of the target gene was amplified by polymerase chain reaction (PCR) and cloned into pCR2.1TOPO, a TA cloning vector (Invitrogen Corp., USA). The insert was then excised from pCR2.1TOPO by digestion with *Sac*I and *Xba*I, and was subcloned into the corresponding sites of pEP185.2, a suicide vector coding for chloramphenicol resistance (Wang & Kushner, 1991). The resulting plasmids—pZM101, pZM102, pZM103, pZM104, pZM105, pZM106, pZM108 and pZM109—were introduced into *Escherichia coli* strain S17λ-*pir* by chemical transformation and transferred into strain ZM100 by conjugation. Exconjugants with a pEP185.2 insertion in the chromosome of strain ZM100 were selected by growth on LB agar plates supplemented with chloramphenicol and nalidixic acid. The primer pairs used for amplification of *sipB*, *ssaV*, *sipA*, *sopB*, *sopE2*, *pipB*, *sseB* and *sseC* are presented in Table 2. The insertion mutations

Table 1. *SE strains and plasmids used in this study*

Strain or plasmid	Relevant genotype	Source or reference
Strain		
SE338	Clinical isolate, PT4	Sheela *et al.* (2003)
ZM100	Derivative of SE338 *nal*^{r}	Present study
ZM101	ZM100 *nal*^{r} *cm*^{r} *sipB::pEP185.2*	Present study
ZM102	ZM100 *nal*^{r} *cm*^{r} *ssaV::pEP185.2*	Present study
ZM103	ZM100 *nal*^{r} *cm*^{r} *sipA::pEP185.2*	Present study
ZM104	ZM100 *nal*^{r} *cm*^{r} *sopB::pEP185.2*	Present study
ZM105	ZM100 *nal*^{r} *cm*^{r} *sopE2::pEP185.2*	Present study
ZM106	ZM100 *nal*^{r} *cm*^{r} *pipB::pEP185.2*	Present study
ZM108	ZM100 *nal*^{r} *cm*^{r} *sseB::pEP185.2*	Present study
ZM109	ZM100 *nal*^{r} *cm*^{r} *sseC::pEP185.2*	Present study
ZM111	ZM100 *nal*^{r} Δ*sipA* (Δ16-606/685)[a]	Present study
Plasmid		
pCR2.1	*Amp*^{r}, *Kan*^{r}, *lacZ*a	Invitrogen
pEP185.2	*cm*^{r}	Wang & Kushner (1991)
pWSK29	*amp*^{r}	Kingsley *et al.* (1999)
pRDH10	*cm*^{r} *sacB*	Kingsley *et al.*, (1999)
pZM101	pEP185.2 carrying a *sipB* fragment	Present study
pZM102	pEP185.2 carrying a *ssaV* fragment	Present study
pZM103	pEP185.2 carrying *sipA* fragment	Present study
pZM104	pEP185.2 carrying so*pB* fragment	Present study
pZM105	pEP185.2 carrying *sopE2* fragment	Present study
pZM106	pEP185.2 carrying *pipB* fragment	Present study
pZM108	pEP185.2 carrying *sseB* fragment	Present study
pZM109	pEP185.2 carrying *sseC* fragment	Present study
pZMSipA	pWSK29 carrying the *sipA* gene	Zhang *et al.* (2002)
pZMSopB	pWSK29 carrying the *sopB* gene	Present study
pZMSopE2	pWSK29 carrying the *sopE2* gene	Present study
pZMPipB	pWSK29 carrying the *pipB* gene	Present study

[a]Data in parentheses are the deleted codons/total number of codon of *sipA*.

Table 2. *Primers used in this study*

Primer	Sequence
SipB-F	ACGCCCGGGAAAAACTCTC
SipB-R	ACTCGCCCCACCGGTAAAAACAGC
SsaV-F2	GCATCTAGAGTCACTCACAATCAGCACA
SsaV-B2	GCACATATCCCGCGAAAACGTCCAGTCC
SipA-F	ATGGTTACAAGTGTAAGG
SipA-R	CAGCCAAAGTTATGTTCA
SopB-F	CCCGTATTTGGTTCTGAATCTCC
SopB-R	AGCCTGAAACTGGTATCCGTGC
SopE2-F1	GCTTCTGAGGGTAGGGCGGTATTA
SopE2-R1	GTTGTGGCGTTGGCATCGTC
PipB-F1	ACGCGGTATACTGGAATGGTTTG
PipB-R1	TGCGTGAGTCAGGTTTGCTTTAGT
SseB-F1	TCAGGAAACATCTTATGGGGAAGT
SseB-R1	GACAGCGTTAAGCTCCTGAGA
SseC-F1	CACTTAAGCAATGTCAGTTGCGT
SseC-R1	GCTCCGGCTTTAACCATTCC
SsaU-F1	GCCGTAAGGAAGGGCAGGTTGTC
SsaU-R1	GCCGCGTCTTCATTTGAGGGTC
SipA-F1-c	GCGAGCTCACGACAACCTGGTAAAAG
SipA-R1-c	GCGAGCTCCTATCAACATCAACGGCA
SopB-F1-c	GCGAGCTCATGATCGCCACTACGTATG
SopB-R1-c	GCTCTAGATCAAGATGTGATTAATGAAG
SopE2-F1-c	GCGAGCTCGTGACAGAAGAACAAAATCC
SopE2-R1-c	GCTCTAGATCAGGAGGCATTCTGAAGAT
PipB-F1-c	GCGAGCTCCTTTAAGTAAATTTTCGCTC
PipB-R1-c	GCTCTAGACTAAAATCTCGGATGGGGGG
SipA-F1-d	AGGCGGCTACTAAAATCC
SipA-R1-d	GCTCTAGATACCTGGCATTATGACGGG
SipA-F2-d	GCTCTAGAGGTCATTACTCATCATCC
SipA-R2-d	CAAGCGAGAGAAAAATACTACAC

were confirmed by Southern hybridization analyses using probes specific for each target gene.

To complement the genetic defect resulting from inactivation of the *sipA*, *sopB*, *sopE2* or *pipB* gene, the entire open reading frame of each gene was amplified by PCR using primers SipA-F1-c/SipA-R1-c, SopB-F1-c/SopB-R1-c, SopE2-F1-c/SopE2-R1-c, or PipB-F1-c/PipB-R1-c. The PCR products were cloned directionally behind the *lac* promoter of pWSK29, a low-copy-number expression vector (Kingsley *et al.*, 1999) to generate plasmids pZM103-c, pZM104-c, pZM105-c and pZM106-c. The sequence and the correct orientation of each target gene in pWSK29 were confirmed by sequencing. The plasmids pZM103-c, pZM104-c, pZM105-c and pZM106-c were introduced into ZM103, ZM104, ZM105 and ZM106, respectively, by electroporation and selection for resistance to ampicillin.

An unmarked deletion of *sipA* (strain ZM111) was constructed by allelic exchange as described previously (Zhang *et al.*, 2002). In brief, DNA regions upstream and downstream of *sipA* were amplified by PCR using primer pairs SipA-F1-d/SipA-R1-d and SipA-F2-d/SipA-R2-d, respectively. The PCR products were ligated, re-amplified, and cloned into pCR2.1TOPO. The insert was excised by digestion with *Eco*RI and subcloned into the vector pRDH10, a *λpir*-dependent suicide vector carrying *sacB* and a chloramphenicol resistance gene (Kingsley *et al.*, 1999). The resulting plasmid was introduced into *E. coli* strain S17*λ-pir* and transferred into ZM100 by conjugation. Exconjugants in which the suicide plasmid was integrated into the ZM100 chromosome were selected on LB agar plates supplemented with chloramphenicol and nalidixic acid. A second recombination event, leading to the loss of plasmid sequences, was selected by growth in sucrose (5%) broth, followed by growth on sucrose agar plates at 30°C. Colonies that were sensitive to chloramphenicol were screened by PCR for deletion of the gene of interest. The unmarked deletion of the *sipA* gene was confirmed by Southern hybridization analyses. The genetic organization of T3SS genes involved in host cell invasion or intracellular survival of SE is shown in Figure 1.

Southern hybridization. Chromosomal DNA was extracted from strains ZM100, ZM101, ZM102, ZM103, ZM104, ZM105, ZM106, ZM108, ZM109 and ZM111 using the DNeasy Blood & Tissue kit according to the manufacturer's instructions (Qiagen Inc., USA). The chromosomal DNA preparations were digested with *Pst*I, subjected to 1% agarose gel electrophoresis, and transferred onto nitrocellulose membrane for subsequent hybridizations. PCR products derived from target genes were used as probes. Probe labelling and hybridizations were conducted using the AlkPhos Direct Labeling and Detection System with CDP-Star (GE Healthcare Life Sciences, USA). The hybridization images were acquired using a Kodak MM2000 gel documentation station.

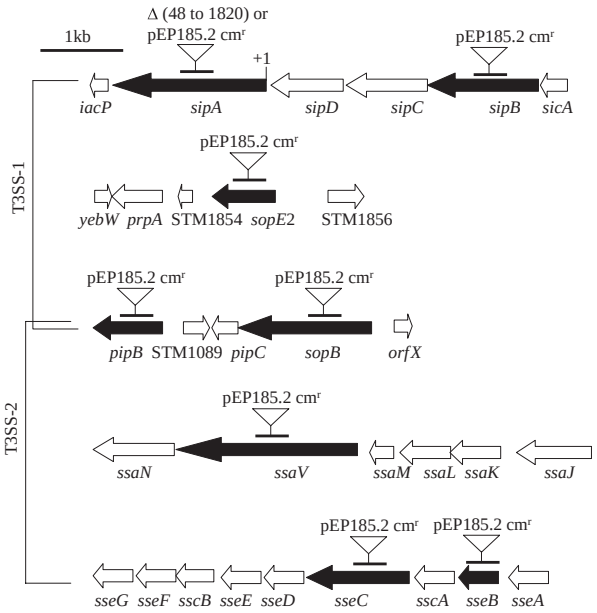

Figure 1. *Mutations in T3SS genes (solid arrows) examined for host cell invasion or intracellular survival of SE. Open arrows, genes in the surrounding regions. Bars, internal fragments of* sipA, sipB, sopB, sopE2, ssaV, pipB, sseB and sseC *that were cloned into pEP185.2 to inactivate these genes. Numbers indicate the position of the deletions relative to the first nucleotide (+1) in the open reading frame of* sipA.

Cell cultures and culture conditions. Primary chicken oviduct epithelial cells (COEC) were prepared essentially as described previously (De Buck *et al.*, 2004). The oviduct tissues of 25-week-old to 28-week-old broiler breeder hens (Ross) were donated by a poultry producer. The salmonella-free status of hens was confirmed by bacterial cultures and PCR. Upon receiving the tissues, the isthmus region of the oviduct was dissected and washed extensively with Hanks balanced salt solution (HBSS) containing 200 U penicillin/ml and 200 mg streptomycin /ml. The isthmal epithelium was treated with collagenase (1 mg/ml), 0.25% trypsin, and 3 mM ethylenediaminetetraacetic acid. Epithelial cells were collected by centrifugation at $125 \times g$ for 10 min and resuspended in Modified Eagle's Minimum Essential Medium (MEM) (American Type Culture Collection, USA) supplemented with 10% heat-inactivated foetal bovine serum, 2% heat-inactivated chicken serum, insulin (0.12 U/ml), and oestradiol (50 nM). The COEC cells were seeded into 96-well tissue culture plates at a density of 2×10^4 cells per well and incubated at 39°C in 5% CO_2 for 48 h. To verify that the cultures were of epithelial lineage, they were examined by immunofluorescent microscopy. In brief, COEC in 96-well tissue culture plates were incubated with an anti-pan cytokeratin (epithelial cell marker) monoclonal antibody at 37°C for 2 h, washed three times, and then incubated with anti-mouse IgG conjugated to fluorescein isothiocyanate at 37°C for 1 h. Staining of cytokeratin in COEC was viewed with an Olympus IX81 FA microscope. Cultures with more than 80% of cells staining for cytokeratin were used in subsequent experiments.

HD11 chicken macrophage cells (Beug *et al.*, 1979) were maintained in RPMI 1640 tissue culture medium (American Type Culture Collection) supplemented with 10% heat-inactivated foetal bovine serum and 2% heat-inactivated chicken serum at 39°C in 5% CO_2. Prior to

infection, HD11 cells were seeded into 96-well tissue culture plates at a density of 2×10^5 cells per well and incubated for 24 h.

Peripheral blood leukocyte-derived macrophages (PBLM) were isolated from Histopague-1077-separated peripheral blood of 25-week-old to 28-week-old broiler breeder hens and were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum and 2% autologous chicken serum. After 2 days of initial culture, adherent monocytes were trypsinized and seeded into 96-well tissue culture plates at a density of 2×10^5 cells per well. The cultures were maintained in supplemented RPMI 1640 medium at 39°C in 5% CO_2 for 7 days to allow monocytes maturation into macrophages. During incubation the medium was changed every 3 days.

Infection of cell cultures. Invasion assays were conducted using the gentamicin protection method as described previously (Hensel *et al.*, 1998). For COEC invasion assays, 50 µl overnight culture of each SE strain was diluted in 5 ml fresh TSB and incubated aerobically at 37°C for 4 h. SE cultures at the logarithmic phase of growth were harvested by centrifugation for 15 min at $1500 \times g$ and resuspended in fresh HBSS without antibiotics. To prepare inocula for HD11 and PBLM, overnight stationary phase SE cultures were harvested by centrifugation at $1500 \times g$ for 15 min and then incubated with 25% normal chicken serum at 37°C for 30 min. The opsonized bacteria were centrifuged again and resuspended in fresh HBSS without antibiotics. The number of bacteria in each culture was determined by measuring the optical density at 600 nm and confirmed by subsequent quantitative culture.

Prior to inoculation, cell cultures were washed three times with warmed HBSS without antibiotics. For each bacterial strain/cell culture/time point combination, 100 µl bacterial suspension containing approximately 4×10^5 colony-forming units (for COEC) or 4×10^6 colony-forming units (for HD11 and PBLM) was added into each of the triplicate wells to reach a multiplicity of infection of 20:1 (bacteria:cell). The inoculated cell cultures were centrifuged at $800 \times g$ for 10 min to synchronize the infection and then incubated at 39°C in 5% CO_2 for 1 h. To remove extracellular bacteria, the infected cell cultures were washed three times with warmed HBSS and incubated in 200 µl HBSS containing gentamicin at a concentration of 100 µg/ml for an additional hour at 39°C in 5% CO_2. After incubation, the infected cells were either lysed in 100 µl of 0.5% Triton X-100 or maintained in supplemented MEM (for COEC) or RPMI 1640 (for HD11 and PBLM) containing 50 µg gentamicin/ml for an additional 3 or 23 h, followed by Triton X-100 lysis. These time points were designated as 1 h post infection (h.p.i.) (T1), 4 h.p.i. (T4) and 24 h.p.i. (T24), respectively. Ten-fold dilutions of the original inocula and cell lysates were plated onto LB agar plates supplemented with appropriate antibiotics and incubated overnight at 37°C for bacterial enumeration. The invasiveness of each strain was expressed as the proportion of the inoculum internalized at T1, and the intracellular survival and replication rate was calculated as the proportion of the number of intracellular bacteria recovered at T4 and T24, respectively, to that at T1.

Cell cytotoxicity assay. At T1, T4, and T24, infected cells and uninfected controls were fixed with 1.85% formaldehyde in phosphate-buffered saline for 15 min, stained with 0.13% crystal violet for 2.5 h, and washed extensively. Absorption was measured using a microplate reader with a 630-nm filter (Dynatech Laboratories). The readings (A630) obtained for the uninfected wells were considered to represent 100% survival, and the cytotoxicity, expressed as the proportion of cell death, was calculated using the formula: $[1 - (A_{630}$ for infected cells / A_{630} for uninfected cells)] $\times 100$, where A_{630} is.

Statistical analysis. The significance of differences in invasiveness and intracellular persistence between wild-type and mutant SE strains, as well as the difference in cell cytotoxicity induced by SE strains, was determined by performing two-tailed Student *t* tests.

Results

Contributions of T3SS-1 and T3SS-2 to SE invasion into COEC, and subsequent survival and replication in COEC. The contributions of T3SS-1 and T3SS-2 to host cell invasion and intracellular replication of SE in COEC were assessed using a gentamicin protection assay. ZM101 (*sipB*), a T3SS-1 mutant impaired in effector translocation, was approximately 10-fold less invasive than the wild-type strain, ZM100 (Figure 2a). Strains harbouring insertion mutations in T3SS-1 effector genes, including *sipA*, *sopB*, and *sopE2,* had about a two-fold reduction in invasiveness compared with ZM100 (wild type). The T3SS-2 mutants, ZM102 and ZM106, carrying mutations in genes encoding a structural protein, SsaV, and an effector protein, PipB, respectively, were also less invasive than ZM100. In contrast to previous findings that inactivation of *sipA* caused a brief delay (15 min) in entry of *Salmonella* Typhimurium into mammalian epithelial cells (Zhou *et al.*, 1999, 2001), ZM103 (*sipA*) was found to be less invasive in COEC than the wild-type SE, ZM100, at 1 h.p.i. To verify the role of *sipA* in SE invasion of COEC, ZM111—a mutant SE strain carrying an unmarked, in-frame deletion in *sipA*—was constructed. Phenotypic analysis indicated that ZM111 was less invasive than ZM103 (Figure 2b). To

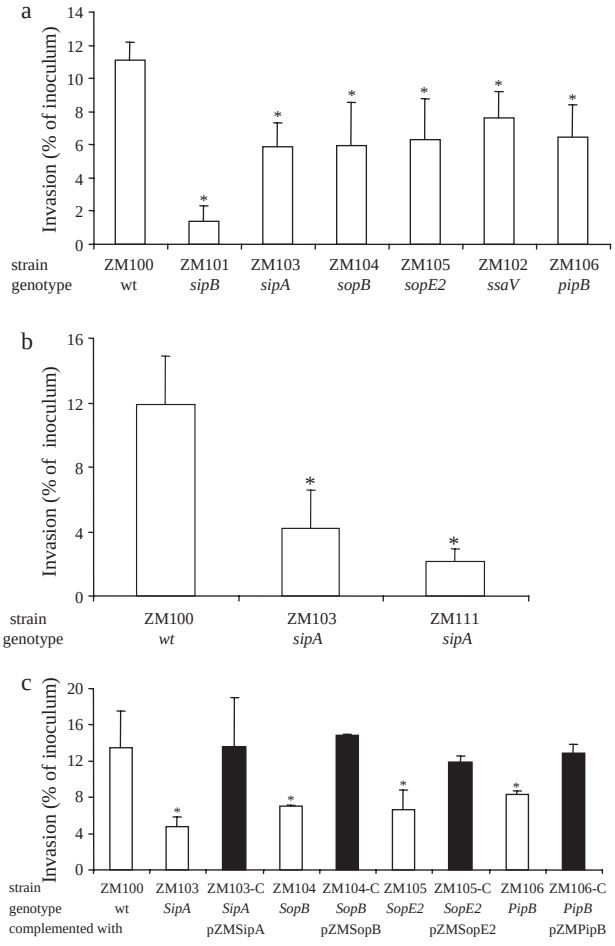

Figure 2. *Invasion of primary chicken oviduct epithelial cells by SE. The invasiveness of each strain was the proportion of the inoculum internalized at 1 h.p.i. 2a: The entry of SE into COEC required both T3SS-1 and T3SS-2. 2b: The sipA mutants were significantly less invasive than wild-type SE at 1 h.p.i. 2c: Invasion defects associated with inactivation of T3SS effector genes were fully complemented by introducing cloned genes into corresponding mutant strains. Data shown (bars) are geometric means of three independent experiments ± standard deviation. *Differences between the invasiveness of mutants and that of the wild-type (wt) SE were significant (*$P < 0.05$*).*

confirm that the decreased invasiveness of T3SS-1 and T3SS-2 mutants did not result from a polar effect due to insertion of pWSK29, a series of complementation assays were conducted. The results demonstrated that the invasion defects associated with mutations in individual effector genes of T3SS-1 and T3SS-2 were fully reversed by introducing the cloned genes on the plasmids pZMSipA, pZMSopB, pZMSopE2 or pZMPipB into the corresponding mutant strains (Figure 2c). These data clearly suggest that SE invasion of chicken oviduct epithelial cells requires the effector proteins translocated by both T3SS-1 and T3SS-2.

We next investigated the contributions of T3SS-1 and T3SS-2 to the survival or replication of SE inside COEC. Following infection with the wild-type SE (ZM100), the number of intracellular bacteria increased about 10% from 1 h.p.i. to 4 h.p.i. and then decreased by about 35% between 4 h.p.i. and 24 h.p.i., although the changes were not significant (Figure 3a). Our results differed from a previous observation that SE continually replicated in COEC between 1 h.p.i. and 24 h.p.i. (De Buck *et al.*, 2004). Two T3SS-1 mutants, ZM101 (*sipB*) and ZM103 (*sipA*), had reduced ability to survive inside COEC in comparison with the wild-type strain ZM100 (Figure 3a). The number of cultivable bacterial cells as well as the survival rates (T4/T1 and T24/T1) for ZM101 and ZM103 were significantly lower than for ZM100. The survival defect of ZM103 (*sipA*) was restored by introducing pZMSipA, encoding the full-length *sipA* gene, into mutant ZM103 (Figure 3b). The number of intracellular ZM104 (*sopB*) at 4 h.p.i., but not 24 h.p.i., was also lower than for ZM100. In contrast to the T3SS-1 mutants, ZM102 (*ssaV*) and ZM106 (*pipB*) showed enhanced proliferation in COEC. From 1 h.p.i. to 4 h.p.i., the number of intracellular ZM102 and ZM106 increased about two-fold (Figure 3a). Of the five effector genes from T3SS-1/T3SS-2 that were examined, *sopE2* had the least influence on the ability of SE to survive or replicate in COEC. To rule out the possibility that differences in the number of intracellular bacteria were caused by variations in the number of viable COEC remaining in the tissue culture plates, cell viability assays were performed. There was no difference in the morphology or the proportion of viable COEC between different treatment groups at 1 h.p.i., 4 h.p.i., or 24 h.p.i. (data not shown).

Phagocytic uptake and persistence of SE in HD11 macrophage. Because T3SS-1 and T3SS-2 were involved in either invasion or intracellular replication of SE in COEC, their contributions to phagocytic uptake and persistence of SE in the HD11 chicken macrophage cell line were evaluated. To minimize T3SS-1 (*sipB*)-mediated macrophage cell death and to enable detection of a T3SS-2-dependent phenotype (Collazo & Galán, 1997; Hensel *et al.*, 1998; Watson *et al.*, 2000), stationary-phase SE cultures opsonized with 25% chicken serum were used to inoculate the HD11 cells. As expected, the viability of HD11 cells following infection was not significantly affected by T3SS-1 or T3SS-2 under the experimental conditions used (data not shown). Our results indicated that the uptake of T3SS mutants by HD11 cells was slightly reduced compared with that of the wild-type strain, ZM100 (Figure 4). Similar numbers of ZM100 and the T3SS-1 and T3SS-2 mutants were recovered from HD11 cells at 4 h.p.i. and 24 h.p.i. (Figure 4).

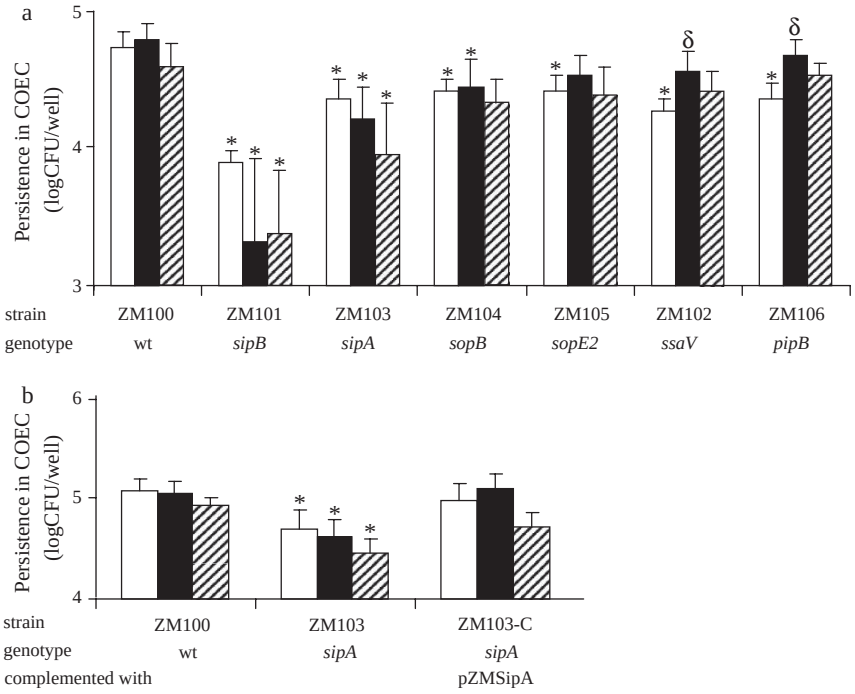

Figure 3. *Differential roles of T3SS-1 and T3SS-2 in SE persistence inside COEC. 3a: Reduced survival and enhanced replication of SE were associated with inactivation of T3SS-1 and T3SS-2, respectively. 3b: Survival defect of ZM103 was restored by introducing pZMSipA carrying the full-length sipA gene into the mutant strain. COEC seeded in 96-well tissue culture plates were infected with SE strains at a multiplicity of infection of 20. Intracellular bacteria were recovered at 1, 4, and 24 h.p.i. Results shown are geometric means of four independent experiments ± standard deviation. Open bar, 1 h.p.i.; solid bar, 4 h.p.i.; hatched bar, 24 h.p.i. wt, wild type.*Difference between the numbers of a mutant strain and of the wild-type SE recovered at a given time point was significant (*$P < 0.05$*). δ, difference between the numbers of a strain recovered at 1 h.p.i. and 4 h.p.i. was significant (*$P < 0.05$*).*

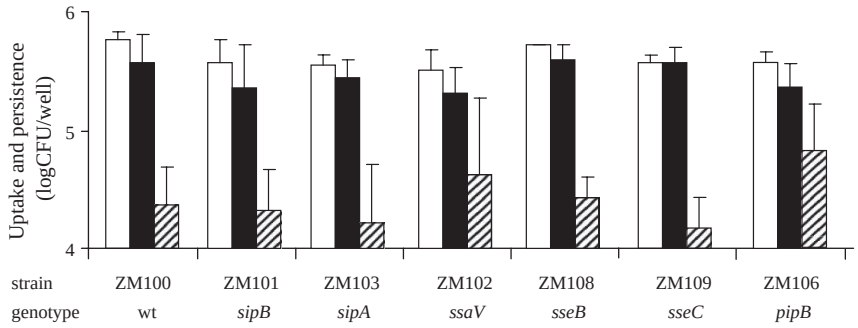

Figure 4. *T3SS-1 and T3SS-2 were not required by SE to persist in HD11 cells. HD11 monolayers in 96-well tissue culture plates were infected with serum-opsonized aerated stationary phase bacteria at a multiplicity of infection of 20. Intracellular bacteria were recovered at 1, 4, and 24 h.p.i. Results shown (bars) are geometric means of three independent experiments ± standard deviation. Open bar, 1 h.p.i.; solid bar, 4 h.p.i.; hatched bar, 24 h.p.i. wt, wild type.*

Phagocytic uptake and persistence of SE in PBLM. To further clarify the roles of T3SS-1 and T3SS-2 in the pathogenicity of SE, the uptake and persistence of selected SE strains in chicken PBLM were determined. The results indicated that the phagocytic uptake of SE by PBLM was not significantly affected by mutations in T3SS genes (Figure 5). During the course of infection, PBLM cells exhibited a greater ability to phagocytose and kill SE, and exhibited signs of more marked cytotoxicity, than HD11 cells. To accurately evaluate the survival ability of each strain, the number of intracellular bacteria was normalized against the number of viable PBLM cells that remained following infection with each SE strain. Between 1 h.p.i. and 4 h.p.i., a survival defect was detected in strains with *ssaV* or *pipB* inactivated, as demonstrated by a reduction of approximately 50% in the number of intracellular ZM102 (*ssaV*) or ZM106 (*pipB*) compared with the wild-type strain ZM100. By 24 h.p.i., there was a 100-fold reduction in the number of viable intracellular bacteria in all strains tested, and no significant difference was detected between wild-type and mutant SE strains. Introduction of pZMPipB, encoding the full-length *pipB* gene, into ZM106 partially repaired the survival defect (Figure 5).The T3SS-1 mutant, ZM103 (*sipA*), had slightly reduced survival compared with the wild-type strain, but the difference between ZM103 and ZM100 (wild type) was not significant.

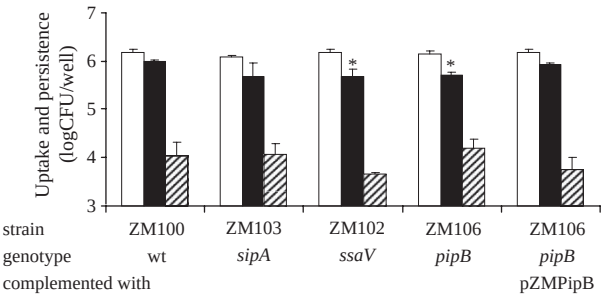

Figure 5. *The T3SS-2 secreted effector, PipB, contributed to the survival of SE in chicken PBLM. PBLM monolayers in 96-well plates were infected with serum-opsonized aerated stationary phase bacteria at a multiplicity of infection of 20. Intracellular bacteria were recovered at 1, 4, and 24 h.p.i. Results shown (bars) are geometric means of three independent experiments ± standard deviation. Open bar, 1 h.p.i.; solid bar, 4 h.p.i.; hatched bar, 24 h.p.i. wt, wild type. *Difference between the numbers of a mutant strain and of the wild-type SE recovered at a given time point was significant (*$P < 0.05$*).*

T3SS-2-dependent PBLM cytotoxicity. *Salmonella* grown to late exponential growth phase, a condition that induces maximal expression of T3SS-1 genes, causes rapid macrophage death due to the activation of cysteine protease caspase1 by SipB (Chen *et al.*, 1996; Monack *et al.*, 1996). Early macrophage cell death is triggered by *Salmonella* invasion, but not phagocytosis (Monack *et al.*, 2001). To avoid SipB-mediated macrophage killing, we used serum-opsonized stationary phase SE cultures to infect chicken PBLM at a relatively low multiplicity of infection (20:1). However, cell death still occurred as early as 1 h.p.i. and throughout the course of infection (24 h). At 1 h.p.i., ZM102 (*ssaV*) infection killed fewer PBLM than infections with the wild-type strain ZM100 and mutant ZM106, but the difference in cell death that resulted from infection with ZM102 and other strains was not significant (Figure 6). At 4 h.p.i., minimal cell death was induced by ZM102 (*ssaV*), whereas death of about 50% and 35% of the PBLM was caused by ZM100 and ZM106, respectively. At 24 h.p.i., nearly 40% of the PBLM cells were killed in a non-T3SS-dependent manner by all strains tested. These data collectively suggest that different mechanisms are involved in the killing of PBLM by SE and that T3SS-2 (non-PipB-dependent) is a major contributing factor in the SE-mediated PBLM death that occurs between 1 h.p.i. and 4 h.p.i.

Discussion

As the first step towards more profound understanding of the molecular mechanisms involved in SE colonization of chicken hosts, we characterized the pathogenic roles of T3SS-1/T3SS-2 effectors during SE infection of chicken oviduct epithelial cells and macrophages. Here we have shown that both T3SS-1 and T3SS-2 contributed to SE invasion of COEC and that this involved genes encoding the effector proteins SipA, SopB, SopE2 and PipB. Among the T3SS-1 effector genes examined, *sipA*, *sopB*, and *sopE*2 were equally important in the entry of SE into COEC, and the *sipA*-associated invasion defect was detectable at 1 h.p.i. Our data differed from previous findings that showed inactivation of *sipA* only caused a 15-min delay in the entry of serovar Typhimurium into host cells (MDCK), and that the *sipA* mutant was as invasive as its wild-type parent strain (Zhou *et al.* 1999,

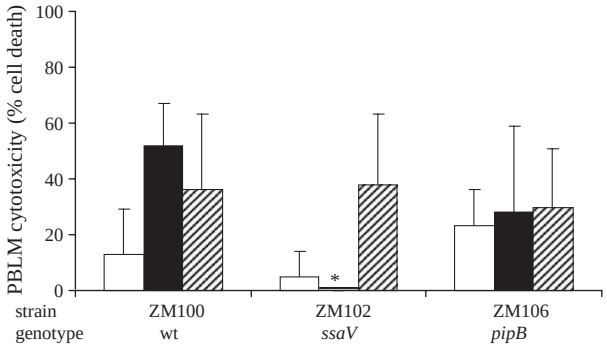

Figure 6. *T3SS-2-mediated PBLM death during the early stages of SE infection. The proportionate cell death was calculated based on the absorbance of infected and uninfected cell cultures at a given time point at 630 nm after crystal violet staining. Data shown (bars) are geometric means of three independent experiments ± standard deviation. Open bar, 1 h.p.i.; solid bar, 4 h.p.i.; hatched bar, 24 h.p.i. wt, wild type. *Proportion of cell death induced by the ssaV mutant and was significantly lower than that induced by the wild-type (wt) strain (*$P < 0.05$*).*

2001; Boyen *et al.*, 2006). While a significant contribution of SipA to mammalian host-cell invasion can only be clearly demonstrated by complementing a non-invasive strain (*sipAsopABDE2*) with the cloned *sipA* gene (Raffatellu *et al.*, 2005), it is apparent that SipA plays a more significant and relatively prolonged role in the entry of SE into COEC. The distinction between our results and those of others indicates that there may be a host-specific or tissue-specific function of SipA, because a *sipA* mutant (ZA10) of serovar Typhimurium is also significantly less invasive to COEC than its parent strain, IR715 (data not shown). In mammalian cells, SipA facilitates invasion by lowering the critical concentration of G-actin and stabilizing F-actin at the site of bacterial entry (Jepson *et al.*, 2001; Zhou *et al.*, 2001). At this time, it is not known why SipA has a prolonged role in salmonella invasion of COEC. The explanation for this phenomenon may lie in differing affinities of SipA for chicken actin and mammalian actin or possibly interactions between SipA and other proteins involved in COEC cytoskeletal remodelling. Nevertheless, the results of this study point to a need to unravel the cellular events triggered by SipA in chicken oviduct epithelial cells. Data from this study also reveal a role for T3SS-1 in the survival of SE inside COEC (Figure 3). The survival defect displayed by ZM103 (*sipA*) was comparable with that of ZM101 (*sipB*). Because a major function of SipB is to translocate effector proteins via T3SS-1, it is likely that the survival defect shown by ZM101 was due to the failure of ZM101 to secrete SipA into COEC. However, we are unable to exclude the possibility that SipB functions as an effector to enhance the survival or replication of SE in COEC. A recent study showed that SipA is exposed on the cytoplasmic face of SCV, from where it promotes the replication of serovar Typhimurium in mammalian host cells (Brawn *et al.*, 2007). Whether the same mechanism is utilized by SE to survive or replicate in chicken oviduct epithelial cells needs to be verified.

The effector proteins of T3SS-2 promote intracellular growth of salmonella by interfering with vesicular trafficking and thus preventing exposure to bactericidal agents, such as reactive oxygen intermediate and reactive nitrogen intermediate in infected macrophages (Shea *et al.*, 1999; Gallois *et al.*, 2001; Jones *et al.*, 2001; Steele-Mortimer *et al.*, 2002). Our COEC infection data demonstrate that reduced invasiveness and enhanced intracellular proliferation of SE are associated with mutations in two T3SS-2 genes, *ssaV* and *pipB*. It has been reported that Typhimurium T3SS-2 null mutants are less invasive than their wild-type parent strain and that this trait coincides with the lack of SipC, a TTSS-1 translocase, in culture supernatant (Hensel *et al.*, 1997). Based on this observation, an interaction between TTSS-1 and TTSS-2 has been proposed. Regardless of this possible interaction, SsaV is a structural protein of T3SS-2, and the decreased invasiveness displayed by ZM102 (*ssaV*) may well be a result of its inability to secrete PipB into COEC. The involvement of the *pipB* gene in SE invasion of COEC contradicts previous findings that this gene is not required by salmonella to invade mammalian epithelial cells (Wood *et al.*, 1998; Knodler *et al.*, 2003). This difference may reflect a host-species-specific function of *pipB*. It is known that *Salmonella* Typhimurium replicates more efficiently in the epithelial cell cytosol than in SCVs, and the maintenance of the SCV membrane is mediated by SifA, an effector protein translocated by T3SS-2 (Beuzón *et al.*, 2002). Thus, the enhanced proliferation of ZM102 (*ssaV*), a T3SS-2 null mutant, in COEC may be due to the migration of SE organisms from SCVs to the cytosol, an environment permissive for bacterial replication. However, this would not explain the increased replication of ZM106 (*pipB*) in COEC, unless the PipB protein is also required for the integrity of SCVs. Further investigation is needed to obtain better insights into host–pathogen interactions in PipB-mediated pathogenicity.

Another finding of this study was that COEC from mature laying hens (25 to 28 weeks old) were able to clear or control SE infection to some extent, as shown by the reduction in the number of SE organisms recovered at the end of the infection experiment. Our data clearly differed from a previous observation that SE replicated approximately 10-fold in COEC between 1 h.p.i. and 24 h.p.i. (De Buck *et al.*, 2004). In this previous investigation, oviduct epithelial cells were obtained from 7-week-old chickens treated repeatedly with oestradiol. The differences in sources of COEC cultures used in the two investigations may explain the different outcomes of the SE–COEC interactions. Indeed, higher levels of antimicrobial peptides, such as the gallinacins, are expressed by the reproductive tissues of laying hens (24 weeks old) than those of young birds (6 weeks old) (Yoshimura *et al.*, 2006). Although the exact mechanism involved in bacterial killing by COEC is unclear, increased production of antimicrobial molecules in the reproductive tract cells of laying hens could contribute to an enhanced defence against SE infection.

Varying degrees of attenuation result from mutations in different T3SS-2 genes (Hensel *et al.*, 1997, 1998). To detect T3SS-2-dependent phenotypes, HD11 cells were infected with three T3SS-2 mutants impaired in effector secretion or translocation, including strains ZM102 (*ssaV*), ZM108 (*sseB*), and ZM109 (*sseC*). However, none of the T3SS-2 mutants had a significant survival or

replication defect, in a sharp contrast to a previous finding that T3SS-2 was required by serovar Gallinarum to survive in HD11 cells (Jones *et al.*, 2001). This difference again suggests serovar-specific properties of T3SS-2. When the selected T3SS mutants, ZM102 (*ssaV*) and ZM106 (*pipB*), were tested in chicken PBLM, small and yet significant survival defects were observed. The different phenotypes demonstrated by the T3SS-2 mutants in HD11 cells and PBLM reflect the different responses of the adapted cell line and primary cells to SE infection. While the reduced survival of ZM102 (*ssaV*) in PBLM confirmed the pathogenic role of T3SS-2 during *S. enterica* infections, the contribution of *pipB* to SE intra-macrophage survival is intriguing. The PipB protein is localized to the SCVs and the salmonella-induced filament (Sif) membrane in mammalian host cells, but its function inside mammalian macrophages is unknown (Knodler *et al.*, 2003; Knodler & Steele-Mortimer, 2005). This protein is not required by serovar Typhimurium to survive in macrophages of mammalian origin (Pfeifer *et al.*, 1999; Knodler *et al.*, 2003; Knodler & Steele-Mortimer, 2005). In a murine typhoid model, inactivation of *pipB* resulted in a delay in the onset of disease (Knodler *et al.*, 2003). In a bovine ligated ileal loop model, the *pipB* mutant induced less inflammation and secretion, but invaded the intestinal epithelium as efficiently as its wild-type parent (Wood *et al.*, 1998). Regardless of its lesser role in the pathogenicity of salmonellae in mammalian hosts, the *pipB* gene has been implicated in intestinal tract colonization of chicks by serovar Typhimurium (Morgan *et al.*, 2004). Here we show that PipB promotes the survival of SE in chicken PBLM, which may be a host-species-specific phenomenon. Although the molecular mechanisms involved in this process remains to be determined, the enhanced proliferation in COEC and the reduced survival in PBLM displayed by the *pipB* mutant suggests a role for PipB in maintaining the integrity of SCV or interfering with intracellular vesicular trafficking in chicken cells. Our data on the contributions of *pipB* to the entry and survival of SE in chicken cells provide a molecular basis for a recent observation that SE carrying a mutation in *ssrA*, a regulatory gene of T3SS-2, is less able to colonize the chicken reproductive tract (Bohez *et al.*, 2008).

Studies have shown that the T3SS-1 effector protein SipB triggers cell death in mammalian macrophages within 45 min of salmonella infection and that T3SS-2 is responsible for the delayed killing of macrophages that occurs after 20 h.p.i. (Chen *et al.*, 1996; Monack *et al.*, 1996, 2001). Here we show that the onset of T3SS-2-dependent PBLM cell death is much earlier than has been reported for mammalian macrophages. Further understanding of the cellular events triggered by T3SS-2 in chicken macrophages may explain the difference in the timing of T3SS-2-mediated cell death in chicken and mammalian macrophages.

In summary, we have characterized the pathogenic roles, both conserved and unique, of several T3SS effector genes in SE infection of chicken cells. It is clear that serovar-specific and host-specific variations exist in the complex interactions of *Salmonella* pathogens and their hosts, and much more investigation is needed to delineate the molecular mechanisms by which SE colonizes chicken reproductive tract.

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